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14. ABSTRACT

We continue to derivatize and test the collagen mimetic peptides (CMPs) for imaging. Our proposed milestones in year two include: (1) Dual radio- and fluorescent labeling of CMPs retaining high-affinity ($K_d \leq 10^{-8}$ M) and specificity for intact and digested collagen (type I) films; (2) Validation of dual-labeled CMPs that display high affinity and specificity for stromal collagens in frozen PCa xenografts; and, (3) Measurement of pharmacokinetics and *in vivo* imaging of dual-labeled CMPs in mouse subcutaneous PC-3 xenograft models. Efforts have been focused on achieving a biologically stable CMP endowed with both gamma-emitting and fluorescent labels (milestone 1). To that end, we have employed several strategies utilizing the previously biologically validated, high affinity basic CMP peptide core, which may contain chelators, covalently bound radioiodine moieties and fluorescent dyes.

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INTRODUCTION

Small collagen mimetic peptide (CMPS) that mimic the amino acid sequence and three dimensional structure of collagen were shown to have specific binding affinity to type I collagen fibers. Although the exact mechanism of binding is not known fully, evidence is accumulating that supports the idea that the CMP is binding to partially denatured domains of natural collagen by triple helical hybridization. Here we use CMP as a collagen targeting agent that will allow imaging of prostate cancer (PCa). Since CMP binds to unstructured collagen domains more readily, it is expected to exhibit selective affinity to metastatic PCa known to contain processed and denatured collagens. This is the first time that the remodeled ECM of tumor microenvironment is targeted for cancer imaging which is an entirely new way to image PCa with a potential to revolutionize the cancer community with respect to imaging and possibly treating PCa and its microenvironment.

BODY

As noted in the abstract, we have employed several strategies utilizing the previously biologically validated, high affinity basic CMP peptide core (**Figure 1**), which may contain chelators, covalently bound radioiodine moieties and fluorescent dyes.

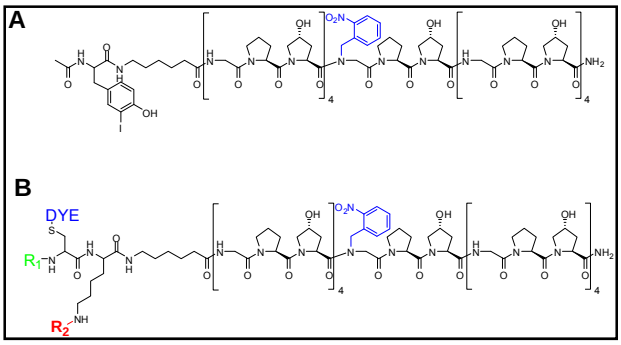


Figure 1. Basic structures of CMPs for radio- and fluorescent dye labeling. Panel (A) shows a photocaged radioiodotyrosyl species with no dye and (B) shows the basic photocaged CMP structure allowing radiolabeling at either lysine 2 or at the amino terminus with chelators for radiometal installment (R_2) or covalent labeling using *p*-iodobenzyl ester (SIB, R_1 or R_2). Cysteine 1 is utilized exclusively for conjugation with maleimide-functionalized dyes.

We first synthesized and tested CMP1, which is depicted in **Figure 1**, panel A to determine whether radiolabeled CMPs displayed significantly altered *in vivo* pharmacokinetics and targeting in the absence of a lipophilic near-IR emitting dye. Ahx-CMP_9 was synthesized with an N-terminal tyrosine and was subsequently radiolabeled with I-125 using the Iodogen method. Radiolabeling was nearly quantitative and the resulting peptide was purified using HPLC. Two immunocompetent adult, female CD-1 mice were injected with purified CMP1 with one dose being photodeprotected prior to injection and the other remaining photocaged as a negative control. **Figure 2** shows longitudinal SPECT-CT imaging of that pair of mice.

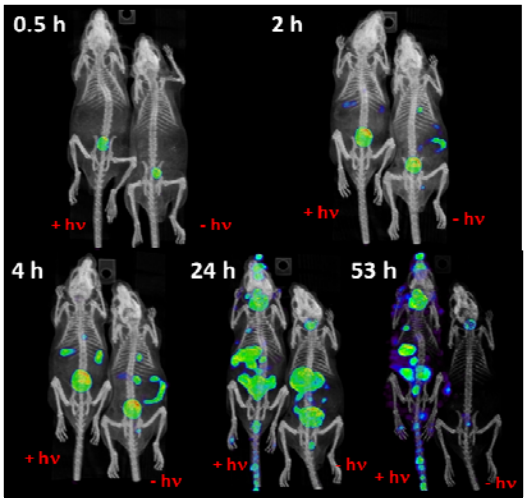


Figure 2. Serial SPECT-CT imaging of CD-1 mice injected with either photodecaged [^{125}I]CMP1 (left mouse, + hν) or still-caged CMP1 (right mouse, - hν) at the indicated times. While iodinate tyrosine is a substrate for dehalogenases *in vivo* over time, it is clear that at least some of the photodecaged labeled CMP1 injected into the left mouse remains in the body by 53 hours post-injection and appears to be localized to the spine, rear and forelegs, and possibly nasal cartilage. The mouse on the right retains only metabolized radioiodine accumulated in thyroid and urinary bladder.

Because our aim is to target the soft-tissue collagens of prostate tumors, we performed a dye labeling experiment using either IRDye800CW (LI-COR Biosciences, Lincoln, NE) or IRDye680RD (also LI-COR Biosciences) to confirm pharmacokinetic or targeting differences we began to observe. The IRDye800CW carries a net charge of -3 at physiological pH and is otherwise highly lipophilic. IRDye 680RD carries a net charge of -2 at pH 7.4 and is less lipophilic. We observed that CMPs labeled with IRDye800CW localize primarily to soft tissue collagens and also ossified bone (data not shown) while CMPs labeled with IRDye680RD appear to localize exclusively to articular cartilage within joints (**Figure 3**).

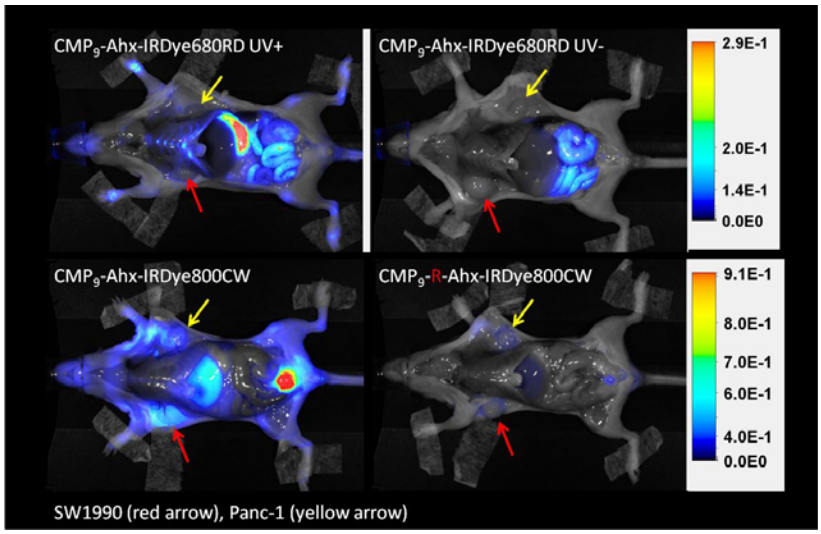
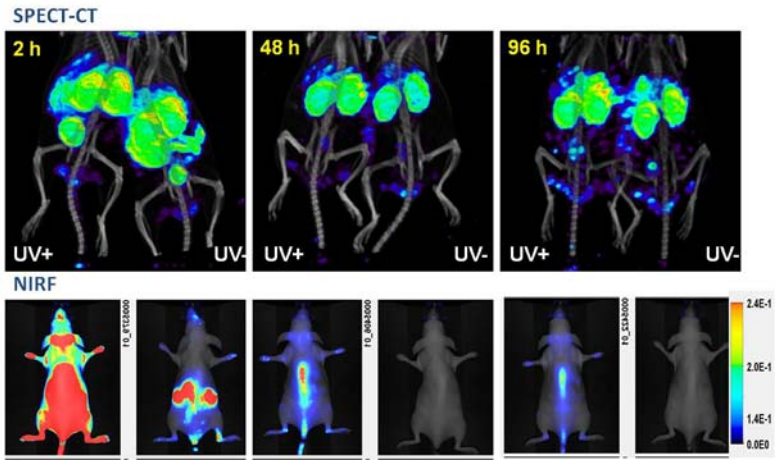


Figure 3. NIRF imaging of tumor-bearing mice with CMPs conjugated to either IRDye680RD or IRDye800CW and imaged at 96 h post-injection. Photodecaged IRDye680RD-conjugated CMP (top left) localizes exclusively to cartilage (wrists, ribs, knees, ankles and toes. GI signal is due to chlorophyll autofluorescence. The same conjugate without photodecaging (top right) does not localize to cartilage or the two subcutaneous tumors (arrows). The mouse injected with photodecaged IRDye800CW conjugate (bottom left) localizes differentially to the tumors (arrows), the surrounding skin, the liver and a small amount of cartilage uptake in knees and ankles. The control scrambled sequence, decaged IRDye800CW conjugate (bottom right) shows only faint uptake within the tumors and liver, reinforcing CMP binding specificity and apparent dye-mediated selectivity.

With the dye selectivity in mind, we further pursued a dual-modality CMP analog containing the dye of choice (we began with IRDye680RD for targeting ease (cartilage) in normal mice). We began with a NHS ester activated benzyl-bridged DTPA chelator, which is easily conjugated to the free epsilon amine of lysine 2 in **Figure 1B** because DTPA will bind In-111 with high affinity and is reported to retain the indium *in vivo* without requiring acidic and heated conditions to chelate the metal. This DTPA, IRDye680RD CMP conjugate (CMP2), was successfully synthesized and purified by HPLC by M. Yu's personnel and was radiolabeled in 100 µM NaOAc, pH 5 for 30 minutes at room temperature with [^{111}In]$InCl_3$ (Nordion Inc., Ottawa ON). Following purification by mini-dialysis (Pierce, MWCO 2000) in PBS, pH 7.5 over 1 h at room temperature, the radiochemical yield was > 90%. Radiochemical purity was assessed by analytical HPLC and was > 95%. Four equivalents of cysteine were added to the formulated [^{111}In]CMP2 in PBS. Two mice were injected with this formulation where one mouse received photodeprotected [^{111}In]CMP2 and the other mouse received still-caged [^{111}In]CMP2 negative control. The mice were then serially scanned using SPECT-CT and NIRF imaging (**Figure 4**). That experiment revealed that the CMP2 was binding to cartilage as expected but only in the fluorescence images. The SPECT-CT scans showed only kidney with a small amount of liver uptake and little uptake in spine. This suggested that the DTPA chelator may not be able to strongly chelate the In-111 *in vivo* over the relatively long 96 hour uptake period.

Next, a p-benzyl-isothiocyanate bridged DOTA conjugate was made to substitute for the DTPA (position R2 in Figure 1B) as a higher affinity chelator (**Figure 5**, CMP3), which will resist transchelation *in vivo*, resulting in a biologically stable dual-labeled peptide. CMP3 was successfully synthesized and purified by HPLC prior to radiolabeling.

Figure 4. Serial SPECT-CT and NIRF imaging of dual-labeled [^{111}In]CMP2. Photodecaged tracer (UV+) and still-caged (UV-) tracer injected mice are shown. NIRF imaging (lower panels) clearly demonstrate the expected cartilage-targeted binding by the decaged tracer only (spine) while the SPECT-CT imaging does not replicate this pattern. The SPECT-CT images show primarily renal excretion and possibly binding since that uptake persists out to 96 h post-injection.



Radiolabeling with [^{111}In]$InCl_3$ occurred in 100 µM NaOAc buffer with heating to 45° C for one hour. Free In-111 was removed by microdialysis cassette against PBS, pH 7.5 for 1 hour. Radio TLC was used to assess radiochemical purity before and after a test-decaging. The presence of multiple radioactive spots after photodecaging suggested liberation of both radiometal, possibly caused by the presence of reductive cysteine quench. The 0.25 equivalents of cysteine added to the vehicle during CMP formulation for injection is to react with and detoxify the resultant benzyl aldehyde produced during decaging. Nevertheless, CMP3 was tested in two

normal SKH mice, who were imaged using both SPECT-CT and NIRF imaging to track both the radiolabel and the fluorescent CMP. **Figure 5** shows the CMP structure and imaging results.

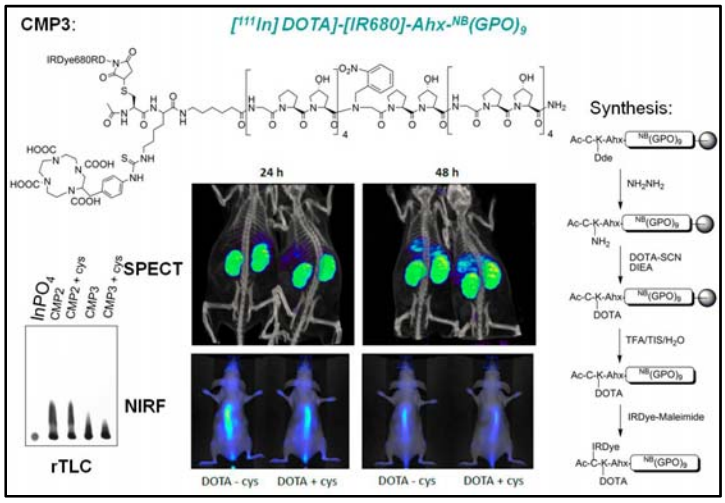


Figure 5. CMP3 structure, synthesis, radio TLC and *in vivo* imaging results. Radio TLC in bottom left shows multiple labeled species following photodeprotection in the presence and absence of cysteine. The DTPA conjugated (CMP2 in lanes 2 and 3) migrate further than CMP3 conjugates, likely reflecting a net -1 charge on CMP2. Spots at the origin may be free radiometal or may represent non-migratory autotriplex CMP following photodecaging.

KEY RESEARCH ACCOMPLISHMENTS

1. Reliable, specific imaging of collagen remodeling with CMPs derivatized with near-infrared fluorescent dye.
2. Imaging with a ^{125}I-labeled CMP, *in vivo*.
3. Deeper understanding of the pharmacokinetics of the CMP-based imaging agents to allow radiolabeling with ^{111}In or a more tractable version of ^{125}I.

REPORTABLE OUTCOMES

We have not generated an imaging-based publication at this time. We are planning for the optimum *in vivo* study, with pharmacokinetically optimized reagents, before publication.

CONCLUSIONS

The fluorescent imaging consistently shows skeletal targeting with the IRDye680RD dual-labeled conjugates and always after decaging and with targeted peptide (ie not scrambled sequence). The SPECT-CT imaging with radioindium-containing chelator conjugates showed no observable evidence of skeletal cartilage targeting despite concomitant NIRF imaging showing fluorescent targeting. This suggests the radiometal is likely being liberated prior to or immediately after injection. Because photocaged CMPs must be deprotected with intense UV light for 5 minutes and that process generates heat, one hypothesis is the heat from decaging (or from thermal melting in uncaged, triplex CMP) coupled with the relatively high pH of PBS (the vehicle used for mouse injections) liberates a significant portion of both DTPA and DOTA-chelated radioindium complex, which is also suggested by the radio TLC data.

Labeling with radioiodine, however, provided evidence of some success with SPECT-CT imaging that is also consistent with concomitant fluorescent imaging. Labeling with N-acetylaated iodotyrosine (CMP1) provided sharp contrast between targeted CMP and scrambled sequence CMP by 53 hours post-injection. While iodotyrosine is a biological substrate for dehalogenases, *p*-iodo-benzoylamide (SIB) is not. We plan to incorporate [^{125}I]SIB at lysine 2, where it will be protected from exopeptidases by the adjacent bulky cysteinyl-IRDye residue. This should result in a chemically and biologically stable dual-labeled CMP (functionalized with either dye) capable of reporting on both soft tissue PCa tumors and bony metastases.